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# Cloning, Characterization, and Expression of a G-Protein-Coupled Receptor from *Lymnaea stagnalis* and Identification of a Leucokinin-Like Peptide, PSFHSWSamide, as Its Endogenous Ligand

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Neuropeptides are known to be important signaling molecules in several neural systems of the pond snail *Lymnaea stagnalis*. Although the functions of these peptides have been studied in many neurons, the nature of the postsynaptic signal transduction is mainly unknown. The cloning and characterization of neuropeptide receptors in *Lymnaea* thus would be very valuable in further elucidating peptidergic pathways. Indirect evidence suggests that these neuropeptides operate via G-protein-coupled mechanisms indicating the presence of

G-protein-coupled receptors as the initial postsynaptic targets. Here we describe the cloning of a neuropeptide receptor from *Lymnaea* and the isolation of an endogenous ligand. This peptide, PSFHSWSamide, belongs to the leucokinin family of peptides, and, thus, this *Lymnaea* receptor is the first example of a leucokinin-like neuropeptide receptor, representing a new subfamily of G-protein-coupled neuropeptide receptors.

**Key words:** neuropeptide receptor; mollusc; CHO-K1; HPLC; calcium; neural networks

Leucokinins comprise a family of peptides that are found in the CNS of insects and have been shown to be involved in diuresis (Hayes et al., 1989; Coast et al., 1990). For instance, leucokinin VIII significantly increases the rates of transepithelial NaCl, KCl, and water secretion in Malpighian tubules of the yellow fever mosquito *Aedes aegypti* (Pannabecker et al., 1993). The leucokinins were first isolated from the cockroach through their ability to induce cockroach hindgut contraction (Holman et al., 1986a,b, 1987a,b); subsequently, leucokinin-related peptides were found in the cricket (Holman et al., 1990) and the locust (Schoofs et al., 1992), all through the use of the hindgut bioassay. To date, 23 members of this family have been isolated: eight from the cockroach, five from the cricket *Acheta domestica*, one from the locust *Locusta migratoria*, three from the mosquito *Culex Salinarius* (Clottens et al., 1993), three from the mosquito *Aedes aegypti* (Veenstra, 1994), and three from the earworm *Helicoverpa zea* (Blackburn et al., 1996). Immunocytochemistry has been used to determine the anatomical location of leucokinins and leucokinin-related peptides in insects, showing then to be in many areas of the CNS (Lundquist et al., 1993; Nassel, 1993; Chen et al., 1994). They have not yet been isolated from any other species, although immunocytochemical studies using antisera raised to insect leucokinins have indicated the presence of leucokinin-like peptides

in the CNS of the parasitic nematode *Ascaris suum* (Smart et al., 1993), the spider *Cupiennius salei* (Schmid and Becherer, 1996), and also in the mollusc *Helix pomata* (Elekes et al., 1994).

It is likely that the leucokinins and leucokinin-related peptides constitute an important group of peptides in insects and invertebrates in general, considering their myotropic actions and involvement in the control of water and electrolyte levels. Further studies on their function are hampered by a paucity of data regarding the postsynaptic sites of action; hence, the identification and characterization of a leucokinin receptor would be of great value with respect to this. The CNS of the pond snail *Lymnaea stagnalis* is one of the best understood systems in terms of defined neural networks and their relation to behavior, especially with respect to neuropeptides (Benjamin and Burke, 1994); thus, we have attempted to use this animal to characterize neuropeptide receptors. Molecular cloning techniques were used to isolate cDNAs encoding neuropeptide receptors from the CNS of *Lymnaea*. One of these cDNAs, designated GRL104, encoded a protein that had features previously described for other neuropeptide receptors. GRL104 was expressed in stably transfected CHO-K1 cells, and the peptide ligand was identified by the novel use of a multistep HPLC approach, together with a functional calcium assay. The peptide sequence was deduced to be PSFHSWSamide. When this peptide was analyzed for sequence homology against a protein database, it showed very high homology to the leucokinin family of peptides, especially leucokinins IV and VI, with five of the last six C-terminal residues being identical to the purified *Lymnaea* peptide. This is the first report of a leucokinin-related peptide being isolated from the CNS of an animal other than insects.

Some of this work has been presented previously in abstract form (Tensen et al., 1994a).

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## MATERIALS AND METHODS

**Animals.** Adult *Lymnaea stagnalis* (shell height 28–34 mm) were bred in the laboratory under standard conditions.

**Molecular procedures.** Standard procedures were performed as described by Sambrook et al. (1989). Restriction enzymes were purchased from NBL (UK) or Boehringer Mannheim (Indianapolis, IN). Synthetic oligonucleotides were synthesized on an ABI 380A synthesizer.

**Polymerase Chain Reaction (PCR).** PCR reactions were performed in 100  $\mu$ l volumes containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 200  $\mu$ M each dNTP, and 5–100 pmol of primers in a Perkin-Elmer (Norwalk, CT) 9600 thermal cycler, using 1 U AmpliTaq (Perkin-Elmer) or 0.2 U of SuperTaq (HT Biotechnologies, Cambridge, UK).

**cDNA library screening.** Total RNA isolated from *Lymnaea* CNS was converted into first strand cDNA using oligo(dT) Dynabeads (Dynal, Oslo, Norway) and reverse transcription. Two primers were synthesized corresponding to DNA sequences within conserved regions of transmembrane (TM) III and TM VI of many G-protein-coupled receptors (Libert et al., 1989) [sense (S1) 5'-CCGGATCCG(CT)(GC)AT(CT)(GA)-(GC)(GC)IT(GT)GAC(CA)G(GC)TA-3'; antisense (A1) 5'-ACGAAT-TCGG(GC)(CA)ICCA(GA)CAGI(GC)(GA)(CT)(GA)AA-3']. These were used in a PCR reaction under the following conditions: denaturation at 96°C for 10 min and then 48 cycles at 94°C for 40 sec, 50°C for 2 min, and 72°C for 3 min. Multiple PCR products were generated, and those of the expected size (400–800 bp) were recovered from an agarose gel with a pipette tip and reamplified in 32 cycles under identical conditions. The total PCR mix was cloned in pBluescript II KS+ (Stratagene, La Jolla, CA) for sequence analysis. Oligonucleotides based on these sequences were used as primers in a PCR-based screening (Bloem and Yu, 1990) of a *LYMNAEA* CNS cDNA library, modified as described previously (Tensen et al., 1994b). Approximately 10<sup>6</sup> independent clones, amplified in 10 aliquots of 10<sup>5</sup> original clones each, were constructed from poly(A<sup>+</sup>) mRNA from *Lymnaea* CNS using a commercial cDNA synthesis [with oligo (dT) priming] and cloning system according to the manufacturer's instructions. To isolate the corresponding full-length cDNA clones, we excised cDNA inserts from these clones *in vivo* as phagemids and sequenced them. DNA sequences were determined from both strands, using the dideoxy chain termination method (Sanger et al., 1977) with denatured double-stranded DNA as a template and using the Sequenase DNA sequencing kit (United States Biochemicals, Cleveland, OH). Automated sequencing was also performed with an ABI 373 automated DNA sequencer. One clone, GRL104, showed high identical amino acid homology (30%) to the neuropeptide Y receptor when the GenBank database was screened.

**Transfection of GRL104 into CHO-K1 cells.** A Kozak optimal ribosomal binding consensus sequence (5' TTTGGATCCGCCATGGCTCAAA-TCGAGTCGATGTCCG 3'; the start ATG is highlighted in bold) was incorporated, using PCR, around the presumed start ATG (Kozak, 1986) of the *Lymnaea* receptor sequence. Then this modified sequence was cloned into the *Bam*HI/*Xba*I sites of the mammalian expression vector pcDNA1/neo (Invitrogen, San Diego, CA), which contains a cytomegalovirus promoter, an SV40 origin of replication, and the neomycin resistance gene. The resulting expression construct, pcD104, was transfected into CHO-K1 cells by lipofection with Lipofectin Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions, and stably expressing CHO-K1 clones were generated by continuous selection with 400  $\mu$ g/ml of the neomycin analog G-418 in DMEM supplemented with 10% fetal calf serum and 1% glutamine.

**Assay for the ligand to GRL104.** CHO-K1 cells stably transfected with pcD104 were resuspended in PBS containing 1 mM CaCl<sub>2</sub> at a concentration of  $2 \times 10^6$  cells/ml. Fura-2-acetoxymethyl ester (Molecular Probes, Eugene, OR) was added to the cells at a final concentration of 1  $\mu$ M. The cells were kept at 4°C for 60 min to allow the Fura-2 ester to penetrate. The cells were washed with PBS three times and then resuspended in PBS containing 1 mM CaCl<sub>2</sub> and 0.1% BSA at a concentration of  $2 \times 10^6$  cells/ml. The cells were kept on ice until the fluorescence assay (a time gap of no more than 10 min). One milliliter of cell suspension was warmed rapidly to 37°C in a quartz cuvette, which was placed in a heated cuvette chamber containing an integral magnetic stirrer. The cells were stirred constantly during the experiments. The fluorescence spectrophotometer (Beckman, Fullerton, CA) was set to excite at 340 nm and monitor emission at 510 nm, and fluorescence changes were recorded on a chart recorder running at 10 cm/min. The slit width was 10 nm, and the response time was two seconds. Dissolved ligands or HPLC fractions were added to the cell suspension; fluorescence responses, if they oc-

curred, were measured immediately. To establish the maximum and minimum amounts of fluorescence in each assay, we added Triton X-100 to achieve a final concentration of 0.1% and then EGTA to achieve a final concentration of 10 mM. Intracellular calcium concentrations were quantitated with equations described by Grynkiewicz et al. (1985).

**Extraction and purification of lymnokinin.** Five hundred CNS were dissected, collected on dry ice, and stored at –60°C until use. They were homogenized in an all-glass Elvehem-Potter homogenizer in 5 ml of 0.1 M acetic acid over ice, boiled for 5 min, and centrifuged at 4°C for 5 min. The supernatant was filtered and applied to a C18 solid-phase extraction column (Supelclean, Supelco, Bellefonte, PA), and bound material was eluted with a mixture of 60% acetonitrile and 7.0 mM trifluoroacetic acid (TFA). The crude peptide fraction was subjected immediately to high-performance gel permeation chromatography (HPGPC), which consisted of applying the crude peptide extract to Protein-Pak columns I-125 and I-300 connected in series (Water Associates, Milford, MA), using a running solvent of 7 mM TFA in 30% CH<sub>3</sub>CN. The liquid chromatographic system (Waters Associates) consisted of an M-720 system controller, an M-730 data module, an M-6000A solvent delivery system, and an M-441 UV/VIS absorbance detector. Thirty fractions were collected, lyophilized, and then resuspended in 500  $\mu$ l of water. Bioactive fractions were pooled and subjected to reverse-phase HPLC (rpHPLC). First we used a Nucleosil C18 column (250  $\times$  4.6 mm), applying a discontinuous linear gradient of water and CH<sub>3</sub>CN in 7.5 mM TFA (10 min 0% CH<sub>3</sub>CN and then 0–60% CH<sub>3</sub>CN over 60 min). Approximately 60 fractions were collected, lyophilized, and redissolved in distilled water. Bioactive fractions were pooled and subjected to a second round of rpHPLC using a narrow bore Nucleosil column (250  $\times$  2.1 mm), applying a discontinuous linear gradient of water and CH<sub>3</sub>CN in 0.05% HCl (10 min 0% CH<sub>3</sub>CN and then 0–60% CH<sub>3</sub>CN over 60 min). Bioactive fractions from this rpHPLC run were rechromatographed with the same column and solvents but with a shallower gradient (12% CH<sub>3</sub>CN over 10 min and then 12–24% CH<sub>3</sub>CN over 40 min) using a Gynotek system that consisted of a 480G gradient pump, a model III channel on-line degasser, and a UVD 320S photo-diode array detector.

**Peptide sequence determination and synthesis.** Amino acid sequences were determined with a pulse liquid automatic sequencer (model 473A, Applied Biosystems, Foster City, CA) as described previously (Li et al., 1993). Peptides were synthesized by Fluorenylmethoxycarbonyl and t-butoxycarbonyl chemistry on an ABI 432A peptide synthesizer.

**Mass spectrometry.** Mass spectral analyses were performed on a Quattro-II triple-quadrupole mass spectrometer equipped with an electrospray source (Micromass). The first quadrupole (ms 1) was used for the mass measurement of the peptide and the parent ion selection for tandem mass spectrometry. For parent ion selection, the first quadrupole was set to transmit a mass window of 2.5 Da. The (M + H)<sup>2+</sup> ion species was selected for fragmentation. The collision cell pressure was set at 3.3 mB throughout the run, and the collision voltage was set to 15 V. Argon was used as the collision gas. The quadrupole analyzer (ms 2) was scanned over the range of 50–1000 mass to charge (*m/z*). The resolution was set to ~1000.

## RESULTS

### cDNA cloning

The degenerate oligonucleotide primers S1 and A1 were used to obtain a PCR product, named GRL104 (see Materials and Methods). GRL104, when analyzed and compared against the GenBank protein database, showed high (25–32%) identical amino acid homology to a number of G-protein-coupled neuropeptide receptors. An oligonucleotide primer specific to GRL104 was used in conjunction with pBluescript forward and reverse arm primers in a PCR reaction on the cDNA CNS library to generate a larger GRL104 PCR product containing the full-length 5' region. If *Lymnaea* neuropeptide receptors are similar to those cloned from other species, then the distance from the exact primer to the 5' end should be in the region of 1000–1500 bp. Bands in this size range were cloned into a pBluescript plasmid and transformed into DH5 $\alpha$ . Plasmids isolated from two colonies were fully sequenced to identify the start methionine of GRL104. A candidate for a start methionine was found in one of the GRL104 clones, and this clone was isolated from the library by PCR screening (Bloem and Yu, 1990). The DNA sequence of the clone GRL104 is shown in Figure 1.

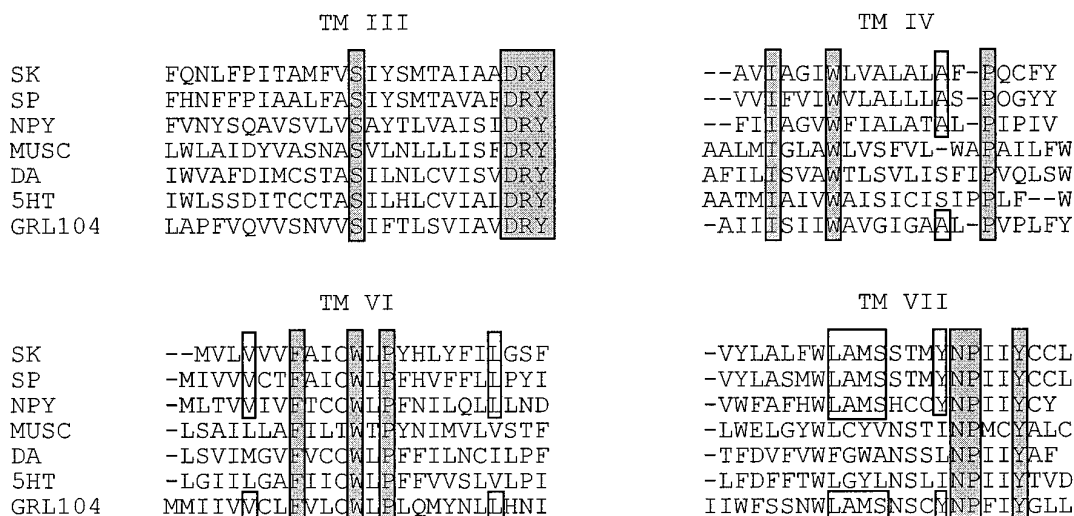
CCTCGGATAATTTAGAGCTGTCGCTTGAACCATGGAACATAATGTGACTTATGTATGAAGC 63  
 CATGAGAGTAGCGGGACCTTTGCCCATGAAACATGGGGATTGTGAGAAGGTACGGGACCTTGT 126  
 GTAACATATTTCTTTGAAGGACAGTTAACTCGTTAATCTGAAGAAAAAGTTACCTGATGACTCT 189  
 TCTTTAAAGGAAATCAAGTTTAGTTGTATGACTCTTAATCGCTGAGGATCAAAATGTAACATA 252  
 CTGGTGAGGCTGCAATGATTGCAACTCTTCTCTTTAGCCAGGGCTAGCGCTCTATCGGT 315  
 TTTGTTTTTTTTTACCCTTTTATTCCTGGAGCTGGATTTAAAGTTTTTTTTTACTTAACATG 378  
 Met 1  
 TCTCAAATCGAGTCGATGTCCGAGCAGGCTGCGGTCTCTTCATCGAGCAGGCCAACAGGAC 441  
 SerGlnIleGluSerMetSerGluGlnAlaAlaValIlePheIleGluGlnAlaAsnGlnAsp 22  
 CTGGACAACGTCAGCGGAATGACGTGAGCAGTTTCTTCTATAACGAAACGACGACCTTGTTT 504  
 LeuAspAsnValSerGlyAsnAspValSerSerPhePheTyrAsnGluThrThrThrLeuPhe 43  
 CCGGGCAGCAACGAGAGCTTCGTGATGCCCTACGACGTGCCACGGGGCTGATCTGCCTCCTG 567  
 ProGlySerAsnGluSerPheValMetProTyrAspValProThrGlyLeuIleCysLeuLeu 64  
 GCGTTCCTGTACGGCTCCATCTCGTGCTGGCCGTCATAGGCAACGGGCTGGTCATACTGGTC 630  
 AlaPheLeuTyrGlySerIleSerLeuLeuAlaValIleGlyAsnGlyLeuValIleLeuVal 85  
 ATCTGTAAGAACAGGAGGATGCACACGGTGACCAACATCTTCATCGCCAACCTGGCCGTGTCG 693  
 IleValLysAsnArgArgMetHisThrValThrAsnIlePheIleAlaAsnLeuAlaValSer 106  
 GACGTTATCATCGGCTTGTTCCTACCTTTTCAGTTCCAAGCGGCCCTTCTGCAGCGCTGG 756  
 AspValIleIleGlyLeuPheSerIleProPheGlnPheGlnAlaAlaLeuLeuGlnArgTrp 127  
 GTGTGGCCAACTTCATGTGCTCGCTGGCCCTTCGTCCAGGTGGTCAGCGTCAACGTCAGC 819  
 ValLeuAlaAsnPheMetCysSerLeuAlaProPheValGlnValValSerValAsnValSer 148  
 ATCTTCACGCTCAGCGTCATCGCCGTGGACCGCTACATCGCCGTCATCCACCGTTCAAGGCC 882  
 IlePheThrLeuSerValIleAlaValAspArgTyrIleAlaValIleHisProPheLysAla 169  
 GGCTGCTCCAAGAAGTCGCGGCCATCATCATCAGCATCATCTGGGCCGTGGGATCGGCGCC 945  
 GlyCysSerLysLysSerAlaAlaIleIleIleSerIleIleTrpAlaValGlyIleGlyAla 190  
 GCGCTCCGGTGCCGCTGTCTACTGGGTGGAGACCTCACCGAGAACAACATCGTGATCCCC 1008  
 AlaLeuProValProLeuPheTyrTrpValGluAspLeuThrGluAsnAsnIleValIlePro 211  
 AGGTGCGACTGGCAGCGCGGCAACTGGCTGGATTTTCACCTGTATTACAACACGCTGCTG 1071  
 ArgCysAspTrpHisAlaProAspAsnTrpLeuAspPheHisLeuTyrTyrAsnThrLeuLeu 232  
 GTGTGTTTCCAGTACCTGCTGCCCTGGTCAATCATCACCTACTGTTACTGCCGATAGCCTGG 1134  
 ValCysPheGlnTyrLeuLeuProLeuValIleIleThrTyrCysTyrCysArgIleAlaTrp 253  
 CATATCTGGGGTCACGGCGACCTGGCGCCACGTCACCACTGAGGACGTACGGGACGTAAC 1197  
 HisIleTrpGlySerArgArgProGlyAlaHisValThrThrGluAspValArgGlyArgAsn 174  
 AAGCGAAAGGTCGTAAAGATGATGATCATCGTCTGCTGCTCTGCTCTGCTGGTGCCG 1260  
 LysArgLysValValLysMetMetIleIleValValCysLeuPheValLeuCysTrpLeuPro 295  
 CTCCAGATGTACAACCTGTGACAACTCAACCGCTCATCAACCACTACCATTACATCAAC 1323  
 LeuGlnMetTyrAsnLeuLeuHisAsnIleAsnProLeuIleAsnHisTyrHisTyrIleAsn 316  
 ATCATCTGGTTTTCGTTCAATTGGCTGGCCATGAGCAACTCTTGCTACAACCTTTTCATCTAC 1386  
 IleIleTrpPheSerSerAsnTrpLeuAlaMetSerAsnSerCysTyrAsnProPheIleTyr 337  
 GGCCTCTGAATGAAAAGTTCAAACGTGAGTTTCACCACTGTTCTGTCATGTGTCCGTGCTGG 1449  
 GlyLeuLeuAsnGluLysPheLysArgGluPheHisGlnLeuPheValMetCysProCysTrp 253  
 AAGGCGCGCTCGACTACTACACGGAGTACTTCTCCGAGGACGCCAACATCTGCGGCGCGCC 1512  
 LysAlaArgValAspTyrTyrThrGluTyrPheSerGluAspAlaAsnIleCysArgArgAla 379  
 AACACCAACGGTCACTGCCCGGCCAACAGGCACGGGCGGTNGGTACAACCTTCTACCGAGACG 1575  
 AsnThrAsnGlyHisCysProAlaAsnArgHisGlyAlaValGlyThrThrSerThrGluThr 400  
 ACAAGAAAGTCTATGCTAAGCAGGTCCAGGTGCAAGCGGACGAGACGGAGACGTCAAACATAT 1638  
 ThrArgLysSerMetLeuSerArgSerArgCysLysArgThrArgArgArgArgGlnThrTyr 421  
 GACGAAAGAAGAGAGACGTATCCTGAAATGACGTCTACGTTTGAGAGGGCGACGGTG 1701  
 AspGluArgArgGluThrSerSer . 429

## Analysis of GRL104

The complete sequence of GRL104 was entered into DNASTAR so that it could be analyzed for open reading frames (ORF). A methionine at position 374 signaled the beginning of the longest ORF, of 1287 bp, which translates into 429 amino acids, and this was taken to

be the coding ORF (Fig. 1). To obtain further information about GRL104, we analyzed the amount of the sequence homology between the full-length amino acid sequence of GRL104 and other G-protein-coupled receptors. The full amino acid sequence resulting from the ORF in the cDNA GRL104 was compared against protein

**Figure 1.** Nucleotide and deduced amino acid sequence of *Lymnaea* GRL104 receptor cDNA cloned in pBluescript BS<sup>+</sup>. A methionine at position 374 indicates the start of the open reading frame of 1287 bp, which translates into 429 amino acids. Putative transmembrane regions are underlined and labeled I–VII. Cysteines suspected of being involved in a cysteine bridge between extracellular loops 2 and 3 are indicated by filled circles. Arrowheads indicate possible N-linked glycosylation sites. The nucleotide sequence for GRL104 has been deposited into GenBank, accession number U84499.



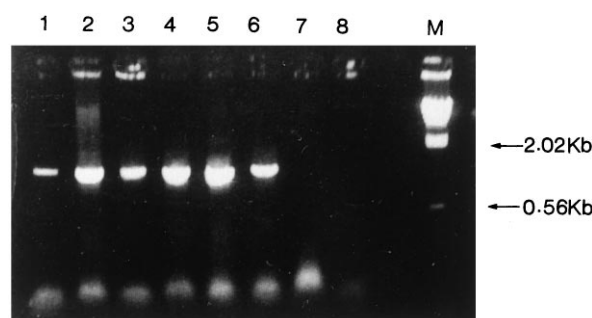
**Figure 2.** Shown are transmembrane (TM) region amino acid alignments between GRL104 and other G-protein-coupled receptors: SK, human substance K receptor (Gerard et al., 1990); SP, rat substance P receptor (Yokota et al., 1989); NPY, human NPY Y1 receptor (Herzog et al., 1992); Musc, human m1 ACh receptor (Peralta et al., 1987); DA, human D1 dopamine receptor (Deary et al., 1990); 5HT, human 5HT1d receptor (Hamblin and Metcalf, 1991). Shaded boxes indicate residues that are conserved among both classical and peptide receptors. Nonshaded boxes indicate residues that are conserved only among peptide receptors.

databases with the DNASTAR program Proscan, which uses the algorithm of Lipman and Pearson to compare amino acid sequence similarities between proteins (Lipman and Pearson, 1985). The protein database search for GRL104 yielded neuropeptide receptors as the top 20 most similar proteins. Specifically, the rat neuropeptide Y (NPY) Y1 receptor was the most similar, with the exact amino acid homology being 32.4% on a 355 amino acid overlap. When the amino acid sequence was analyzed for its hydrophobicity profile (Kyte and Doolittle, 1982), it was found that there were seven regions of hydrophobicity that could be transmembrane domains. There were several domains that had particularly high homology to the rat NPY receptor; these areas closely correlated with the hydrophobic, and thus possibly the transmembrane, domains. In addition to the seven transmembrane domains, GRL104 has a very short extracellular region of only 45 amino acids. At the C-terminal cytosolic end of the receptor there is an 89 amino acid stretch.

Comparisons were made between some of the transmembrane regions in a selection of classical neurotransmitter receptors, neuropeptide receptors, and this *Lymnaea* receptor GRL104. This alignment is shown in Figure 2. GRL104 shows sequence homology to both classical and peptide G-protein-coupled receptors, e.g., the serine and aspartate arginine tyrosine triplet in TM III, the isoleucine and proline in TM IV, the phenylalanine tryptophan and proline in TM VI, and the tryptophan, the asparagine proline doublet, and the tyrosine in TM VII. Further sequence homology is present between GRL104 and neuropeptide receptors alone in that there is a conserved leucine in TM VI, a conserved alanine in TM IV, and a conserved tyrosine in TM VII. These alignments indicated that GRL104 was likely to be a G-protein-coupled neuropeptide receptor.

#### Generation of stable CHO-K1 cell lines

GRL104 was modified as described in Materials and Methods, and the resulting plasmid, pcD104, was used in transfection experiments. CHO-K1 cells were transfected with pcD104, and nine clones were picked for further experiments. To confirm that the plasmid pcD104 had been integrated into the CHO genomic DNA, we analyzed some of these cell lines by PCR. Sense and antisense primers at the



**Figure 3.** PCR analysis of CHO cell lines stably transfected with pcD104, using primers specific to pcD104. Lanes 1–6 contain PCR products from CHO cell lines KC2, KC4, KC5, KC6, KC7, and KC9. Lane 7 is a negative control using untransfected CHO-K1 cells as template. Lane 8 is a control PCR reaction with no template. Lane M contains size markers generated from  $\lambda$  bacteriophage cut by *HindIII*.

extreme 5' and 3' ends of the ORF of pcD104 were used to attempt to amplify the receptor DNA sequence from the cell lines. All the CHO lines tested, KC2, KC4, KC5, KC6, KC7, and KC9, gave PCR products of ~1300 bp, which is the expected size for the GRL104 transcript (Fig. 3). From this it could be inferred that the transfected CHO cells had incorporated pcD104 into the CHO genome. These clones were now called GRL104 CHO cell lines, because pcD104 was carrying GRL104 receptor DNA.

#### Expression of GRL104 in mammalian cells and identification of the endogenous ligand

On binding to ligands, many G-protein-coupled receptors, including peptide receptors, link via G-proteins in a second messenger cascade, resulting in an increase in intracellular calcium concentration. This can be used to assay for ligands to GRL104. The first stage was to challenge the GRL104 CHO cell lines with a CNS peptide extract from *Lymnaea*. Peptides from 500 *Lymnaea* CNS were extracted and purified through a C18 solid-phase extraction column. All nine GRL104 CHO cell lines, together with untransfected CHO cells, were loaded with Fura-2 and challenged with an amount of crude

**Table 1.**

Species	Peptide name	Amino acid sequence	Reference
<i>Lymnaea stagnalis</i>	Lymnokinin	PS <b>F</b> H <b>S</b> W <b>S</b> -amide	This paper
<i>Leucophaera maderae</i>	Leucokinin I	DPA <b>F</b> NS <b>W</b> G-amide	Holman et al., 1986a
	Leucokinin II	DPG <b>F</b> SS <b>W</b> G-amide	Holman et al., 1986a
	Leucokinin III	DQG <b>F</b> NS <b>W</b> G-amide	Holman et al., 1986b
	Leucokinin IV	DAS <b>F</b> H <b>S</b> W <b>G</b> -amide	Holman et al., 1986b
	Leucokinin V	GSG <b>F</b> SS <b>W</b> G-amide	Holman et al., 1987a
	Leucokinin VI	pES <b>F</b> H <b>S</b> W <b>G</b> -amide	Holman et al., 1987a
	Leucokinin VII	DPA <b>F</b> SS <b>W</b> G-amide	Holman et al., 1987b
	Leucokinin VIII	GAD <b>F</b> Y <b>S</b> W <b>G</b> -amide	Holman et al., 1987b
<i>Acheta domesticus</i>	Achetakinin I	SGAD <b>F</b> Y <b>P</b> W <b>G</b> -amide	Holman et al., 1990
	Achetakinin II	AY <b>F</b> SP <b>W</b> G-amide	Holman et al., 1990
	Achetakinin III	ALP <b>F</b> SP <b>W</b> G-amide	Holman et al., 1990
	Achetakinin IV	NFK <b>F</b> NP <b>W</b> G-amide	Holman et al., 1990
	Achetakinin V	A <b>F</b> H <b>S</b> W <b>G</b> -amide	Holman et al., 1990
<i>Locusta migratoria</i>	Locustakinin	A <b>F</b> SS <b>W</b> G-amide	Schoofs et al., 1992
<i>Culex salinarius</i>	CDP I	NP <b>F</b> H <b>S</b> W <b>G</b> -amide	Clottens et al., 1993
	CDP II	NNANV <b>F</b> Y <b>P</b> W <b>G</b> -amide	Clottens et al., 1993
	CDP III	TKYVSKQ <b>F</b> FS <b>W</b> G-amide	Clottens et al., 1993
<i>Aedes aegypti</i>	I	NSKYVSKQ <b>K</b> FYS <b>W</b> G-amide	Veenstra, 1994
	II	NPFHAY <b>F</b> SA <b>W</b> G-amide	Veenstra, 1994
	III	NNPNV <b>F</b> Y <b>P</b> W <b>G</b> -amide	Veenstra, 1994
<i>Helicoverpa zea</i>	Helicokinin I	Y <b>F</b> SP <b>W</b> G-amide	Blackburn et al., 1995
	Helicokinin II	VR <b>F</b> SP <b>W</b> G-amide	Blackburn et al., 1995
	Helicokinin III	KVK <b>F</b> SA <b>W</b> G-amide	Blackburn et al., 1995

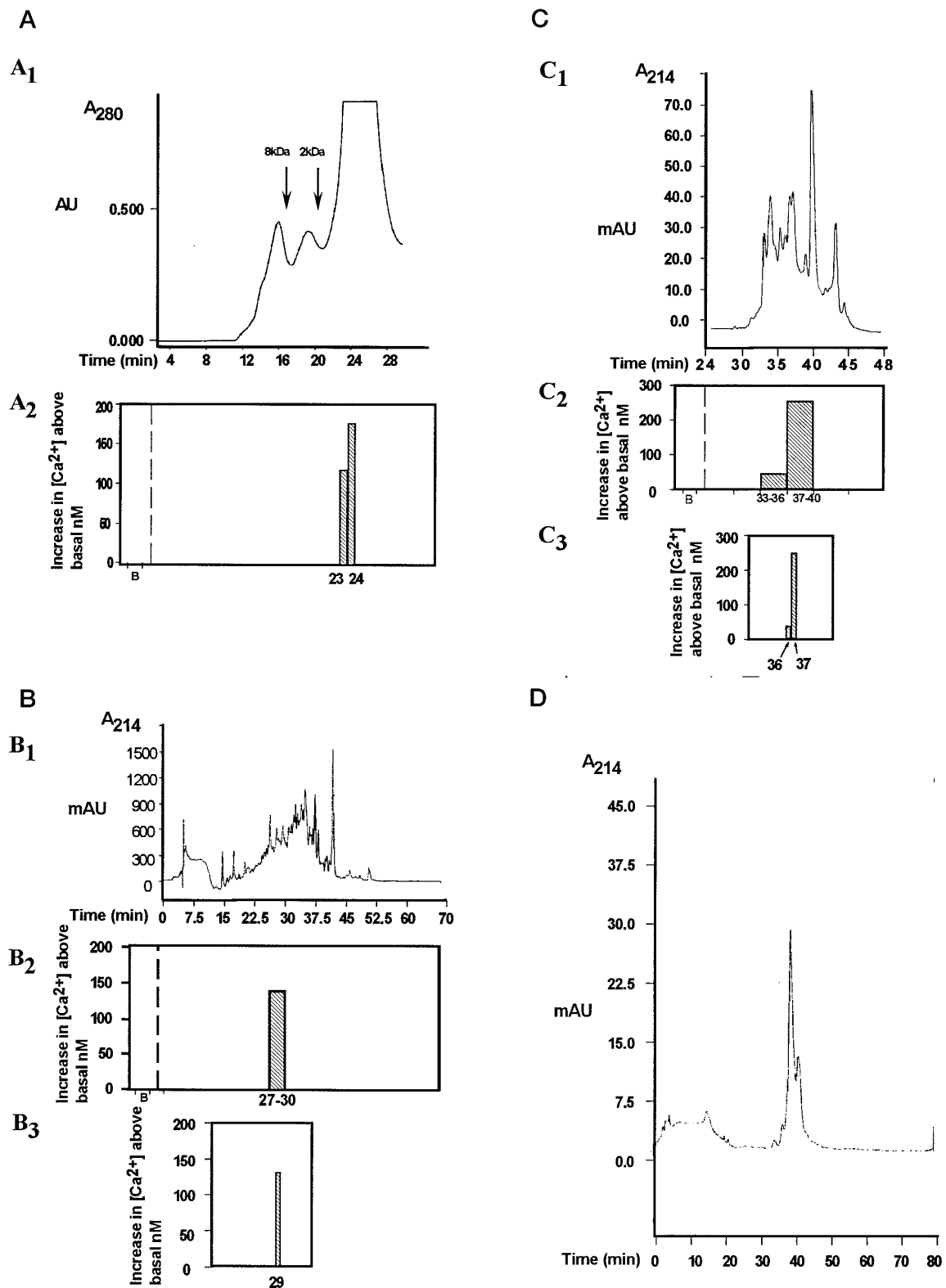
Amino acid sequences of purified lymnokinin and comparison with the leucokinins and leucokinin-related peptides that have been isolated and sequenced from various species. Amino acids that are conserved throughout the family are in **bold**.

peptides equivalent to two CNS. Changes in intracellular calcium were monitored by studying changes in fluorescence. Five of the nine clones responded to the peptide extract, although the other four did not respond. Untransfected CHO cells did not respond to *Lymnaea* CNS peptide extracts. These results suggested that in over 50% of the stable cell lines, where the receptor construct had incorporated into the CHO genome, it was transcribed and, subsequently, translated. Moreover, the results demonstrate that the GRL104 receptor in CHO cells was able to couple to a second messenger system, which had the effect of increasing intracellular calcium. The CHO clone GRL104 KC6 was used in subsequent assays because, of the nine GRL104 CHO stable cell lines, it yielded the largest rise in intracellular calcium when challenged with the CNS peptide extract.

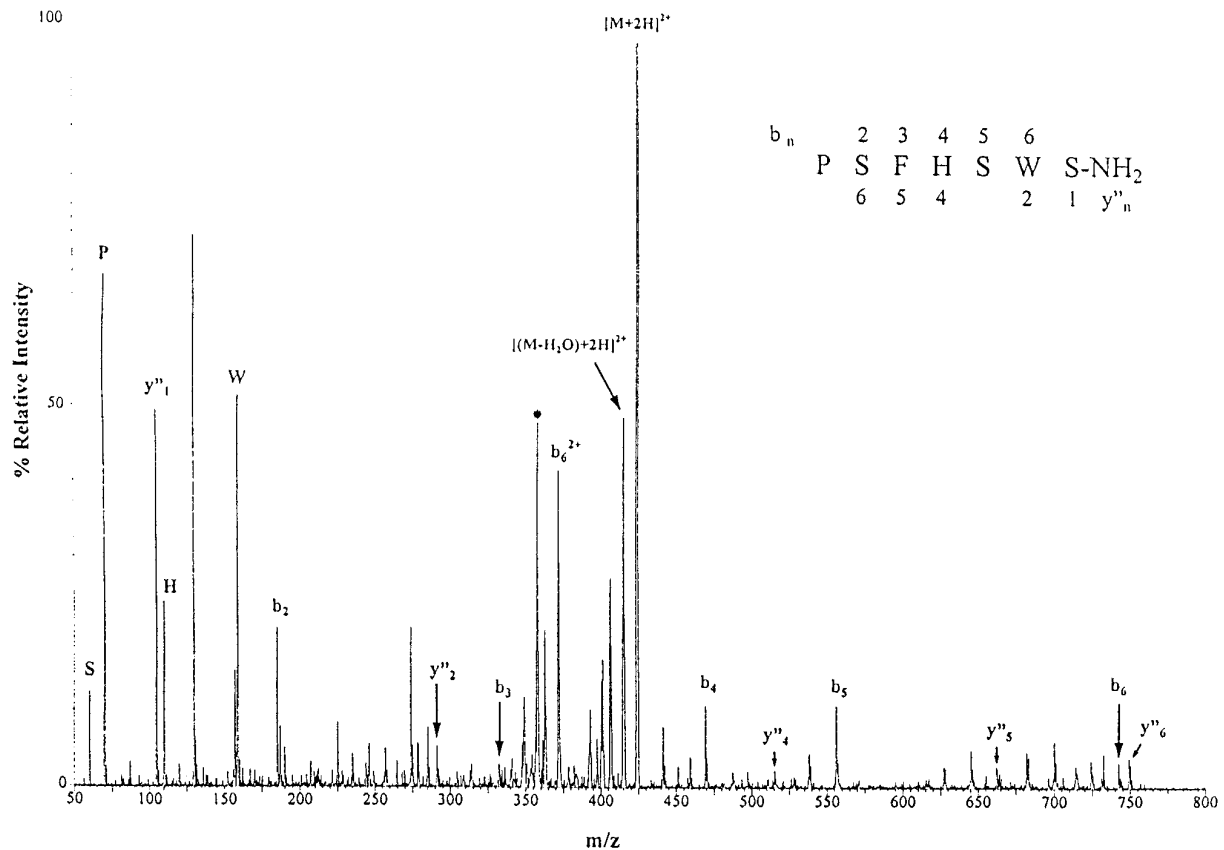
Figure 4 shows the four steps of the HPLC procedure. Fractions 23 and 24 from the gel permeation separation were bioactive in the calcium assay (Fig. 4A) and so were combined and run on the rpHPLC column in step 2 (Fig. 4B). Fraction 29 was positive and further purified in step 3 on a reverse-phase HPLC column (Fig. 4C). Fraction 37 from this run generated a rise in intracellular calcium concentration, and fraction 36 gave a much smaller rise. A final rpHPLC purification run (step 4, Fig. 4D) was performed on pooled fractions 36 and 37 (from step 3), which resolved into two peaks from which it was possible to obtain peptide sequence from both peaks. Edman sequencing revealed one of the peptides to be GDPFLR (the large peak). It is very likely that this peptide represents the previously reported GDPFLRFamide, in which the C-terminal amino acid was washed off from the sequencing filter and cannot be detected. The other peptide (the small peak) was deduced to have the sequence PSFHSWX. As with GDPFLRFamide, the first six residues could be assigned unambiguously; however, the final residue in this peptide was evident only as a

small peak (again as with GDPFLRFamide the C-terminal residue also may have been washed off) and could not be assigned with complete confidence. The sequence PSFHSW was run through a peptide database to establish whether it had any homology to known peptides. This search produced a family of eight peptides, all eight amino acids long, called the leucokinins (Table 1); therefore, we designated the *Lymnaea* peptide lymnokinin. In an attempt to confirm the sequence of lymnokinin, we isolated and sequenced a new batch of peptide. On this occasion closer inspection of the sequencing data indicated the presence of a (C-terminal) serine residue. To ascertain the sequence of the peptide unequivocally, we subjected it to tandem mass spectrometric analysis. Figure 5 shows that the peptide is PSFHSWSamide.

One of the potential ligands, GDPFLRFamide, was unable to stimulate GRL104 even at micromolar concentrations (data not shown). Synthetic lymnokinin, on the other hand, was able to stimulate CHO cells expressing GRL104 at physiological concentrations, and a dose-response experiment was performed (Fig. 6). Lymnokinin had an EC<sub>50</sub> of 1.14 nM, which is similar to other neuropeptide potencies. For instance, SP has an IC<sub>50</sub> of ~1 nM (Hershey and Krause, 1990) for the NK-1 receptor. Because it seemed likely that lymnokinin was indeed a member of the leucokinin family, it was possible that other members of the family also were able to elicit calcium responses in CHO cells expressing GRL104. Three commercial preparations of the insect leucokinins were tested on KC6. Leucokinin IV and leucokinin VI, both of which have five of the last six C-terminal amino acids in common with lymnokinin (Table 1), were able to elicit an increase in calcium concentration in GRL104 KC6 CHO cells at 100 nM or above, i.e., a 100-fold lower potency than lymnokinin, whereas leucokinin V, which only has three amino acids in common with lymnokinin (Table 1), was less potent, being



**Figure 4.** Purification of the endogenous ligand for GRL104. All assays were performed on a CHO cell line that had been stably transfected with pcD104 cDNA (CHO cell line GRL104 KC6). The *top* of each panel, A–C, gives the chromatogram for the HPLC purification indicated. A<sub>1</sub>, HPGPC fractionation of an extract of 500 *Lymnaea* brains; B<sub>1</sub>, rpHPLC fractionation of combined fractions 23 and 24 from A; C<sub>1</sub>, rpHPLC fractionation of fraction 29 from B. D, rpHPLC fractionation of combined fractions 36 and 37 from C. The *bottom* of each panel, A–C, shows the effect of the indicated HPLC fractions on intracellular calcium levels in the CHO cell line GRL104 KC6, using 2, 4, and 8 CNS equivalents for A<sub>2</sub>, B<sub>2</sub>, B<sub>3</sub>, C<sub>2</sub>, and C<sub>3</sub>, respectively. *x*-Axis numbers indicate HPLC fraction number. The calcium increases were calculated as increases in concentration above basal levels. On each HPLC fractionation, A<sub>2</sub> shows the two fractions (of 33) that were active (mean of two determinations), B<sub>3</sub> shows the fraction (of 54) that was active (mean of two determinations), and C<sub>3</sub> shows the two fractions (of 45) that were active (one determination only to conserve enough of the fraction for the next stage of HPLC, seen in D).

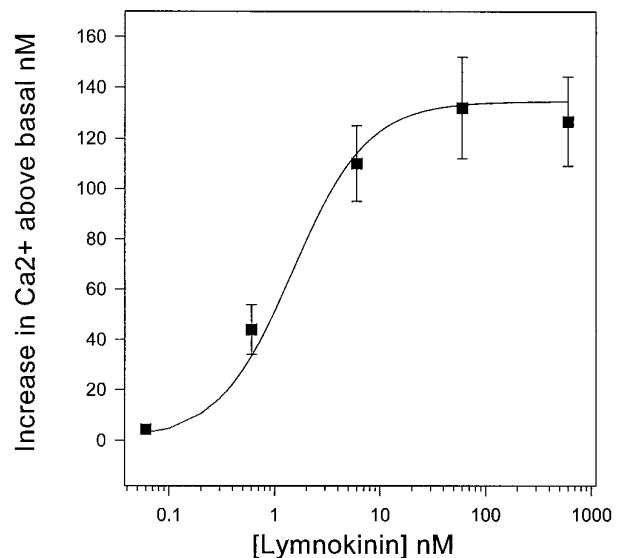


**Figure 5.** Tandem mass spectrometric analysis of lymnokinin. Averaged daughter ion spectra of the purified lymnokinin, generated from the doubly charged parent ion species  $(M + 2H)^{2+}$  of  $m/z$  424 Da. The Roepstorff nomenclature is used to identify fragment ions (Roepstorff and Fohlman, 1984). The  $y''$  ions are formed by charge retention on C-terminal fragments, and the  $a$  and  $b$  ions are formed on N-terminal fragments. The measured protonated mass of the peptide [846.8 Da, as detected in stage 1 (ms 1) of the tandem MS analysis; data not shown] as well as the  $y''$ ,  $a$ , and  $b$  ion series are in perfect agreement with the calculated protonated masses of the peptide PSFHSWSamide (846.9 Da as detected in ms 1; data not shown) and the corresponding  $y''$ ,  $a$ , and  $b$  ion series. The asterisk represents  $a_6^{2+}$ ,  $x$ -axis;  $m/z$  is mass to charge ratio.

able to elicit a response at  $1 \mu\text{M}$  or above (data not shown). On the other hand, PSFHSWGamide, which is identical to lymnokinin apart from the Gamide (which is present in the leucokinins), was as potent as lymnokinin with an  $\text{EC}_{50}$  of 1.2 nM (data not shown). Several other peptides (VIP, FLRFamide, FMRFamide, APGWamide, vasopressin, substance P, substance K, elodisin, and neuropeptide Y) were tested on GRL104 but were unable to elicit any calcium response.

## DISCUSSION

The leucokinin family of peptides (Table 1) has been the focus of considerable interest since they were first isolated from the cockroach and, subsequently, in other insects such as the cricket, locust, and mosquito. They have two main physiological effects on insects; one is to increase hindgut motility in cockroaches (Holman et al., 1986a,b, 1987a,b), and the other is an involvement in insect diuresis (Hayes et al., 1989; Coast et al., 1990). It has been shown that leucokinins have an effect on the transepithelial voltage and the fluid secretion of isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti*, and this effect is speculated to be mediated by an increase in chloride ion permeability of epithelial membranes (Hayes et al., 1989; Pannabecker et al., 1993). Leucokinin-like peptides have effects on the stomatogastric nervous system of the crab *Cancer borealis*; this work shows that individual leucokinins were able to excite the pyloric rhythm and also the DG neuron (which is involved in the gastric mill rhythm), suggesting the presence of an as yet uncharacterized endogenous leucokinin-like peptide in this species



**Figure 6.** Dose-response curve of the increase in intracellular calcium in GRL104 KC6 CHO cells elicited by synthetic lymnokinin. The calcium increases were calculated as increases in concentration above basal calcium levels. Each data point is the mean of three separate determinations. Error bars are  $\pm$  SD.



(Blitz et al., 1995). In *Helix*, an antibody to leucokinin I stained many neurons in the CNS, including interneurons and neurons that are known to innervate peripheral organs, indicating that leucokinins play a wide role in the CNS of molluscs (Elekes et al., 1994). The identification and characterization of the molluscan leucokinin-related receptor GRL104 described here is an important advance in mapping leucokinin neuronal pathways and understanding the physiological function of this family of peptides in molluscs and, potentially, in other invertebrates. Work is currently in progress to localize, in alternate sections of the *Lymnaea* CNS, the relationship between the lymnokinin receptor-expressing cells (using *in situ* hybridization) and lymnokinin (using an antiserum raised against leucokinin IV).

The G-protein-coupled superfamily of receptors (Probst et al., 1992) is composed of a number of subfamilies, one of which is the peptide receptor family. Members of this group include, for instance, the NPY receptors and the tachykinin receptors, which comprise further subfamilies of receptors. GRL104 was most similar to neuropeptide receptors, especially to the rat NPY receptor with 32% identical sequence homology. This amount of homology indicates that GRL104 is very likely to be a neuropeptide receptor, but not a subtype of any known neuropeptide receptor. This, in conjunction with the fact that none of the tachykinins or NPY or any other peptide tested (except leucokinins) were able to mobilize calcium in CHO cells transfected with GRL104, indicates that GRL104 is the first example of a new group of G-protein-coupled neuropeptide receptors.

Of the peptides tested, only PSFHSWSamide (lymnokinin) and PSFHSWGamide were able to activate GRL104 at the nanomolar level. We consider that lymnokinin is the endogenous ligand to GRL104 because it was isolated from the CNS of *Lymnaea*, but we cannot rule out that PSFHSWGamide also may be present in the CNS of *Lymnaea* and a ligand for GRL104. Furthermore, it is possible that other presumably lymnokinin-like peptides are in the CNS of *Lymnaea* that are also ligands for GRL104. Efficacious peptides present in the CNS at very low levels would not be isolated using the above HPLC approach in which only 500 *Lymnaea* CNS were used; it may be that, as in the case of the leucokinins, lymnokinin may be just one of a family of lymnokinin-like peptides. It is interesting to note that members of the leucokinin group of peptides also were able to activate GRL104. Two obvious candidates were leucokinin IV and leucokinin VI, because they have five of the last six C-terminal amino acids in common with the deduced sequence of lymnokinin. These peptides activated GRL104 at 500 nM. Apart from the C-terminal residue, the only differences between lymnokinin and leucokinin IV and leucokinin VI are that the latter two peptides have one extra amino acid and that the amino acid at position seven is an alanine for leucokinin IV and a serine for leucokinin VI, whereas for lymnokinin it is a proline. One of the other members of the leucokinin peptide family, leucokinin V, was tested on GRL104, eliciting a functional response only at  $10^{-6}$  M. This comparatively lower potency is perhaps not surprising considering that only three amino acids were in common (Table 1). It is probable that the other peptides shown in Table 1, apart from acetabulin V and culekinin-depolarizing peptide (CDP) I, which share similar C-terminal regions with lymnokinin, also would elicit a functional response in the micromolar range. In the light of the Gamide being invariant in the leucokinin peptide family, PSFHSWGamide was also synthesized and tested for functional efficacy with respect to GRL104. The fact that PSFHSWGamide has a very similar potency to lymnokinin indicates that the C-terminal residue may not be critical in that it can be Gamide or Samide. This is perhaps not too surprising, because glycine and serine are very similar. Other peptides with little or no

C-terminal sequence homology to lymnokinin were unable to elicit any functional response. These data seem to suggest that lymnokinin N-terminal residues are important for increased potency when coupling to GRL104, with the C-terminal residues perhaps playing a greater role in binding to the receptor. Further evidence indicating the importance of the N-terminal region of neuropeptides with respect to functional potency has been provided previously by structure–activity studies with leucomyosuppressin, a 10-amino-acid-long peptide isolated from cockroach, which is able to inhibit spontaneous contractions of visceral muscles (Nachman et al., 1993). A clear relationship was found between C-terminal peptide content and potency, with the last five C-terminal amino acids being the minimum size for eliciting a functional response. As more residues were added from the C-terminal end, potency increased. Binding studies with both N- and C-terminally truncated lymnokinin analogs are needed to determine whether N-terminal residues are responsible for the increased potency and the C-terminal end for binding to GRL104. Although not examined in the present work, the full dose–response characteristics of the leucokinins with respect to GRL104 also would be of interest regarding the relationship between peptide structure and function.

An apparent difference by a factor  $\sim 50$  is evident between the potencies of the leucokinin-like peptides in the hindgut and Malpighian tubule assays, and lymnokinin in the calcium assays, with CHO cells expressing GRL104 receptor protein. For instance, the  $ED_{50}$  for acetabulin-I in the Malpighian tubule assay is  $7.5 \times 10^{-11}$  M (Coast et al., 1990), whereas the  $EC_{50}$  of lymnokinin with respect to GRL104 is 1.14 nM. This difference may indicate a combined hormonal/neurotransmitter role for lymnokinin, as has been suggested for the leucokinins (Muren et al., 1993). The fact that GRL104 was isolated from a CNS cDNA library is in agreement for its being the receptor for lymnokinin when this peptide is acting as a neurotransmitter in the CNS of *Lymnaea*, and, as has been mentioned previously, the  $EC_{50}$  of lymnokinin with respect to GRL104 is in the range found for other peptide neurotransmitters.

Here we have described the cloning from *Lymnaea* of a neuropeptide receptor, GRL104, for which the endogenous ligand lymnokinin, a leucokinin-like peptide, has been isolated. GRL104 represents the first example of a new subfamily of neuropeptide receptors, the leucokinin-like peptide receptors. Also presented here is the first example of the isolation of a leucokinin-like peptide in an invertebrate other than an insect. Molecular biological techniques such as PCR have enabled many G-protein-coupled receptor sequences, characterized as such purely on homology to known receptors, to be isolated. Many of these receptors are, however, “orphans,” because the nature of the endogenous ligands is unknown. Recently, an approach using HPLC fractions on receptor-expressing cell lines was used to identify a novel opiate ligand to an orphan G-protein-coupled receptor, using a cAMP assay (Meunier et al., 1995; Reinscheid et al., 1995). The approach described in the present work (reported briefly previously, Tensen et al., 1994a), which also uses multistep HPLC but this time uses as the functional response an increase in intracellular calcium, is a more general method for isolating an endogenous ligand to a suspected G-protein-coupled receptor in that both cAMP and PKC second messenger pathways result in an increase in intracellular calcium. This approach should be valuable in identifying the ligands to other orphan receptors.

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